THE CULTURE OF VOLVOX AUREUS EHRENBERG

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Introduction

A number of methods for the culture of *Volvox* are described in the literature, but none of these has been consistently successful in our laboratory. Other investigators have had a similar experience. S. O. Mast (personal communication) and his students have tried these methods time and again over a period of years without securing permanent cultures.

Hartmann (1921) was unsuccessful in culturing *Volvox* although he was able to maintain cultures of *Eudorina elegans* for many months. Knoke's many experiments (1924) over a two-year period were likewise without positive results. After extensive analyses of natural waters containing *Volvox* in large numbers, Uspenski and Uspenskaja (1925) devised an inorganic salt solution (based on Knop's medium) in which *Volvox aureus* Ehrenberg and *V. globator* Linnaeus were cultured bacterium-free for periods of fifteen and four months respectively. These workers showed that a favorable concentration (0.5–1.0 mg. l.) of iron was a decisive factor in the production of healthy cultures, a deficiency of iron resulting in gradual deterioration, an excess of iron producing a poisoning of the *Volvox* colonies. Soil extract or decoction was used by Mainx (1929) who secured rich permanent cultures of *V. aureus* far superior to those obtained with synthetic solutions, including that of Uspenski and Uspenskaja. Pringsheim (1930) reached similar results in experiments with *V. globator* and *V. aureus*, but he likewise was unable to maintain these organisms for any length of time in the Uspenski medium where their growth was slower and of shorter duration than in soil extract medium. Lefévre (1932) states that he secured a clone culture of *V. aureus* in a nutritive solution (derived from that of Czurda) consisting largely of inorganic salts in pond water to which a few fragments of *Sphagnum* were added. About 300 descendant colonies were produced in some ten days, but he does not say whether this clone was maintained for any appreciable length of time. Mixed cultures of Volvocales and other plankton species, which included *V. aureus*, *Pandorina morum* Bory, and *Eudorina elegans* Ehrenberg, were maintained for many months.

Johansen (1940) lists six solutions which produce luxuriant growth of *Volvox*, *Gonium*, *Pandorina*, and *Eudorina*, namely certain soil solutions; 0.05 per cent Benecke's and 0.05 per cent Knop's solutions; and 1.5 per cent Detmer's agar (apparently after Bold, 1936). He also gives a medium which is identical in composition with that of Uspenski.

It would appear from the foregoing that the continuous culture of *Volvox* is a relatively simple and exact matter, and that a variety of media are available for this

[1] I gratefully acknowledge the friendly interest and encouragement in this work shown by the late Dr. S. O. Mast, emeritus professor of zoology, The Johns Hopkins University.

purpose. Nevertheless, preliminary experiments indicated that this is not the case. Soil decoctions vary widely in composition with the soil used, and inorganic salt solutions may not give rich, permanent cultures.

The purpose of the present paper is threefold: (1) to describe a culture method for *V. aureus* which has given consistent results for eighteen months in the continuous culture of the organism; (2) to reduce this method to one which is readily duplicable; and (3) to indicate by experiments the probable complexity of the nutrition of this organism.

Materials and Methods

V. aureus was secured from a pond on the property of the Carolina Biological Supply Company near Elon College, North Carolina, where it occurs throughout the year, appearing intermittently in large numbers.

In all of the experiments ordinary finger bowls (4½ inches ×2 inches) were employed as culture dishes. Two hundred cc. of medium were dispensed in each of the bowls which were then placed in a hot air oven and pasteurized. Inoculations were made after the medium had cooled to room temperature (approximately 21° C.). All cultures were illuminated by the light through a west window, some direct sunlight falling on them during part of the afternoon. Throughout the winter months the low light intensity was supplemented by radiation from a Sylvania fluorescent lamp (Type HF–150S).

Inoculations were made with ordinary medicine droppers which had been previously boiled. No attempt was made to exclude bacteria from the cultures, which however showed little evidence of bacterial action. All other possible contaminants such as protozoa and algae were absent.

The salts used in the preparation of inorganic media were either Baker's Analyzed or Merck's Reagent. The peptone and beef extract were Difco, the creatine was Pfanstiehl cp., the lactic acid was Baker's Analyzed, and the uric acid was Coleman and Bell's Reagent. In all cases stock solutions were made up and then diluted to proper concentration in the preparation of media.

The commercial fishmeal (commonly used as fertilizer) was obtained from Ballard Brothers, Willis Wharf, Virginia. The spring water used in most of the experiments was secured from a surface spring on the property of the Carolina Biological Supply Company. It is referred to as local spring water. The Huckleberry spring water came from a spring by that name near Durham, North Carolina. Distilled water was supplied by a Stokes Automatic Water Still.

Experiments and Results

Experiments with inorganic salt solutions

Modified Knop's medium as recommended by Bold (1936) and Johansen (1940) was prepared and dispensed into finger bowls. Five cultures were inoculated with several hundred colonies of *Volvox*. The cultures were examined daily, but no growth or reproduction was evident. The colonies gradually lost their rich green color and fell to the bottom of the bowl where they remained motionless, and finally deteriorated. Although the experiment was repeated a number of times the result was always the same.

Since Uspenski and Uspenskaja (1925) cultivated *V. aureus* and *V. globator* in an inorganic medium continuously over periods of fifteen and four months respectively, attempts were made to secure similar results with their medium. The composition is as follows:

KNO_3	0.025 gm.
$MgSO_4$	0.025 gm.
$Ca(NO_3)_2$	0.100 gm.
KH_2PO_4	0.025 gm.
K_2CO_3	0.0345 gm.
$Fe_2(SO_4)_3$	0.00125 gm. (added every 10 days)
Distilled water up to 1,000 cc.	

Whereas Uspenski and Uspenskaja worked with bacterium-free cultures and used Leningrad glass culture dishes, the present experiments were conducted in ordinary glass finger bowls containing pasteurized medium. The initial pH was found to be 7.6 which is in exact agreement with these authors.

Five cultures were prepared. Each was inoculated with a single large *Volvox* colony bearing eight daughter colonies. Only one culture showed any evidence of growth and reproduction about a hundred pale green colonies being evident after several weeks. These were permitted to concentrate at one side of the bowl, removed with a medicine dropper, and used as inoculum for five subcultures. Observation over a period of four weeks showed no evidence of growth and reproduction. Gradually the colonies lost their motility, became paler in color, and finally disintegrated. Although the experiment was repeated a number of times, the results were negative.

It was noted that the ferric sulfate had a tendency to be precipitated in some of the cultures shortly after they were pasteurized. Believing that failure of the experiment might be due to a lack of the iron salt, it was repeated with the exception that $FeCl_3$ was used instead of $Fe_2(SO_4)_3$; but, although the former salt remained in solution somewhat better than the latter, the end result was the same.

Experiments with media containing organic materials

Soil decoctions. Several samples of local garden soils were obtained, extracted, and diluted according to the methods of Mainx (1929) and Pringsheim (1930). Although numerous attempts with varied concentrations of soil extract medium were made to secure cultures comparable to those described by these workers, success was not realized. In most instances the organisms rapidly deteriorated and died. In several cases multiplication occurred for a time, but efforts to maintain these cultures through subcultivation failed.

Peptone, beef extract, milk, and urine. Various concentrations of these substances in spring water ranging from 5 mg./l. to 200 mg./l. were tested without securing growth and reproduction for more than a week or so. The first cultures of *Volvox* in beef extract medium (5 mg./l.) exhibited an astounding rate of multiplication for several weeks, thousands of colonies being produced. Subcultures, however, were poorer. Gradually the colonies became paler in color, and after six weeks the third set of subcultures died out. Some evidence of growth was secured in peptone medium (5 to 10 mg./l) but *Volvox* colonies quickly deteriorated in all-concentrations of urine and milk media, in which a considerable growth of bacteria

was evident. Even though all of the culture media contained $FeCl_3 \cdot 6H_2O$ (0.5 cc. 1 per cent sol./l.), this failed to prevent the cultures from dying out.

Commercial fishmeal. An extract of commercial fishmeal was prepared by adding 200 mg. of fishmeal to a liter of spring water and heating to 80–90° C. The mixture was shaken well and filtered (Reeve filter paper, No. 201). The slightly straw-colored filtrate was dispensed in five finger bowls which were then pasteurized. Several hundred *Volvox* were inoculated into each of these. After two weeks one of the cultures was teeming with colonies. Five subcultures from it produced thousands of the organisms. Subcultivation was continued every two weeks, but after six weeks all of the descendant cultures suddenly died out.

This experiment was repeated in exactly the same way except that the cultures were aerated daily by bubbling air through the medium, but the end result was the same.

Recalling that Uspenski and Uspenskaja (1925) had emphasized the importance of iron in the nutrition of *Volvox,* fishmeal extract was prepared as in the previous experiments. To each liter 0.5 cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$ was added. This gave a concentration of the salt of 5 mg./l. or of 1.03 mg. Fe/l. This amount of Fe is slightly greater than the optimum (0.5–1.0 mg./l.) given by Uspenski and Uspenskaja, but it was felt that some of the Fe would be bound by organic substances in the medium and so reduce the available supply. The amount of Fe remaining unbound would be within the optimal range.

Five cultures were set up and inoculated from a clone culture that had been established in fishmeal extract several weeks earlier. This culture contained no iron other than that already present in the spring water used in its preparation.

In approximately two weeks excellent cultures resulted. From these cultures others were established which were in no way inferior. Thus, this clone of *Volvox* has been maintained in continuous culture with undiminished vigor for a period of eighteen months.

Following the recommendation of Uspenski and Uspenskaja, only half as much iron (¼ cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$) was added in winter as in summer. Since the initial concentration of iron was rather high, iron was not added every ten days in summer and once a month in winter as they suggested. The initial pH of the fishmeal medium was consistently 7.6. Over a three-week culture period it was found to fluctuate around this value.

Fishmeal medium, prior to the addition of ferric chloride, is faintly straw colored but clear. After twenty-four hours it becomes slightly cloudy, perhaps through bacterial action. No membrane forms at the surface, however, and in a few days the medium becomes clear.

No attempt has been made to count the total number of colonies in a single culture, but the number surely runs up to several thousand. Cultures reach a maximum population in from two to three weeks depending largely on variations in light intensity and temperature. Over a period of eighteen months reproduction has been asexual; sexual reproduction has not been observed.

Extracts of different strengths in which the amount of fishmeal varied between 50 and 1,000 mg./l. were tested, but 200 mg./l. seemed to give the largest populations of *Volvox.* Therefore, all stock cultures were maintained in media prepared with that amount of fishmeal.

Composition of fishmeal extract

Since *V. aureus* flourished in a medium prepared from spring water, fishmeal, and ferric chloride, it seemed desirable to determine the essential components with a view toward simplification. In this connection the following questions are pertinent: Can a spring water of known chemical composition be substituted for the local spring water? Can spring water be replaced by either tap water or distilled water? Can Uspenski's medium be used in place of spring water? Are the inorganic salts alone in fishmeal, along with those in spring water, sufficient to support the culture of *Volvox?* Does the fishmeal extract contain an appreciable amount of protein, and if so, how important is this in the medium? How important are other extractives?

Water component of fishmeal extract. Seven lots of five cultures were prepared in which the following media were used:

1. Fishmeal + local spring water + $FeCl_3 \cdot 6H_2O$
2. Fishmeal + Huckleberry spring water + $FeCl_3 \cdot 6H_2O$
3. Fishmeal + tap water + $FeCl_3 \cdot 6H_2O$
4. Fishmeal + distilled water + $FeCl_3 \cdot 6H_2O$
5. Fishmeal + Uspenski medium
6. Fishmeal + Uspenski medium ($FeCl_3 \cdot 6H_2O$ used instead of $Fe_2(SO_4)_3$)
7. Local spring water + $FeCl_3 \cdot 6H_2O$

In all cases 200 mg. of fishmeal and ½ cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$ were used.

Each culture was inoculated with ten large *Volvox* colonies of approximately equal size, each colony containing eight daughter colonies.

Volvox flourished in media 1 and 2, but little or no growth occurred in 3, 5, 6, and 7. Large numbers of colonies were produced in medium 4. These were pale green in color, becoming paler with each subcultivation, and finally were moribund after four transfers (68 days). Cultures in media 1 and 2 were maintained for many months and were in a flourishing state when finally discarded. Repetition of the experiment gave substantially the same results.

This experiment clearly shows that the chemical composition of the water used in fishmeal medium is very important in the culture of *Volvox*. Tap water may not be nutritively deficient but toxic, for it has been observed that paramecium, hydra, and other invertebrates do not survive long in the local unchlorinated, artesian water. Distilled water is obviously deficient in certain essential salts, although the lack is not felt for some time. Huckleberry spring water can be substituted for the local spring water since it can supply these salts in adequate amounts. It is interesting to note that the Uspenski medium, prepared to include either ferric sulfate or ferric chloride, failed to support growth and reproduction of *Volvox*, even when fortified with the extractives of fishmeal. Finally, fishmeal supplies important nutritive substances without which cultures of *Volvox* soon perish.

Fishmeal component. Obviously fishmeal is a very complex material. Therefore, no extensive series of experiments was contemplated to determine what substances in fishmeal are so vital in the nutrition of *Volvox*. The following experiment was performed to make clear what class or classes of substances (inorganic

salts, proteins, other extractives) in fishmeal contribute to the growth and reproduction of *Volvox*. Four culture media were prepared according to the schema:

1. Fishmeal ash + local spring water + $FeCl_3 \cdot 6H_2O$
2. Fishmeal + cold local spring water + $FeCl_3 \cdot 6H_2O$
3. Fishmeal residue + hot local spring water + $FeCl_3 \cdot 6H_2O$
4. Washed fishmeal + local spring water + $FeCl_3 \cdot 6H_2O$

One gram of fishmeal was completely incinerated in a chemically clean crucible and the resulting ash shaken well with one liter of spring water. The mixture was heated to 80–90° C. and filtered while hot. Two hundred cc. of this solution were diluted to one liter with spring water in order to secure an inorganic salt concentration comparable to that of regular fishmeal extract.

Another gram of fishmeal was added to a liter of spring water, shaken well, and filtered. Two hundred cc. of the filtrate were diluted with spring water to one liter to give a solution of cold-water extractives approximating the concentration of these substances in regular fishmeal extract.

The residue on the filter paper was added to a liter of spring water and heated to 80–90° C. and filtered while hot. Two hundred cc. of the filtrate were diluted with spring water to one liter. Theoretically this solution contained hot-water extractives roughly equivalent to their concentration in regular fishmeal extract.

The residue on the filter paper was dried and weighed. It had lost one-fourth of its weight. This amount (0.25 gm.) was shaken well with a liter of spring water to form a suspension.

One-half cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$ was added to each liter of culture medium. Five cultures were prepared from each of the four media, pasteurized, and allowed to cool to room temperature. Each culture was inoculated with five large *Volvox* colonies, every one of which contained eight daughter colonies in about the same state of development.

Examination of the cultures over a period of three weeks gave no evidence of growth in media 1, 3, and 4. Medium 2 on the other hand gave excellent cultures which were in no respect inferior to stock cultures maintained on regular fishmeal extract. Subcultures, which were carried for several months, showed no sign of deterioration whatsoever and probably could have been maintained indefinitely.

This experiment demonstrates, therefore, that the readily soluble cold-water extractives of fishmeal complement the inorganic salts of spring water and the ferric chloride to produce a nutritionally complete medium; that the soluble salts in fishmeal ash medium do not meet all of the growth requirements of *Volvox;* and that hot-water extractives and proteins of fishmeal have little or no importance in the culture of *Volvox*.

It might be supposed that proteins would be present in the cold-water extract fraction of fishmeal, but the usual tests for protein were negative.

Fish extracts

Commercial fishmeal consists largely of the pulverized remains of marine fishes, and therefore contains all of the chemical constituents of bone, muscle, skin, viscera, etc. The question as to what part of a fish, if any one part, provided the important nutritive substances in the culture of *Volvox* was investigated as follows:

Several butter-fish (*Poronotus triacanthus* Peck) were secured at a local fish market. These were washed in distilled water. Small quantities (about 10 gm.) of skin, muscle, and bone (vertebral column) were removed very carefully in order not to include any of the adjacent tissues. The three portions were placed in a small amount of distilled water in three separate flasks and boiled until practically all of the water had evaporated. They were then dried on filter paper in a dry air oven. One-half of each portion was separately shaken with ether from time to time for a period of four hours in order to remove lipoids, after which treatment the ether was removed by filtration. The skin, muscle, and bone residues were dried on filter paper for forty-eight hours. The remaining half of each portion of skin, muscle, and bone received no further treatment.

Two hundred mg. each of skin, skin extracted with ether, muscle, muscle extracted with ether, bone, and bone extracted with ether were separately heated with one liter of spring water to 80 – 90° C. and filtered while hot. One-half cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$ was added to each liter of filtrate. Five cultures were prepared from each type of filtrate and inoculated with numerous *Volvox*.

The results are summarized below:

1. Skin medium—colonies died within a few days
2. Extracted skin medium—colonies died within a few days
3. Muscle medium—good cultures; subcultured for three months
4. Extracted muscle medium—excellent cultures; subcultured for three months
5. Bone medium—colonies died within three weeks
6. Extracted bone medium—some growth and reproduction; subcultures unsuccessful

The experiment was repeated using Huckleberry spring water instead of the local spring water. The results were essentially the same with the exception that colonies in the extracted bone medium died out within a week.

The experiment shows that, of the three tissues tested, muscle alone contains an adequate quantity of the substances so vital to the continuous culture of *Volvox*. Furthermore, the conclusion is reached that the lipoids are of no value to *Volvox;* on the contrary they appear to exert a depressing influence as is indicated by the larger populations of extracted muscle medium cultures.

No attempt is made here to deny that connective tissue associated with fish muscle may contribute as much or more than muscle to the nutrition of *Volvox*. It would be next to impossible to separate the two tissues to determine this point. However, in experiments which follow the assumption is made that it is the muscle that is important and not the connective tissue.

Since the butterfish is marine and the associated salts of sea water might conceivably have something to do with the results obtained, experiments were carried out with the fresh-water sunfish (*Lepomis gibbosus* Linnaeus) in order to check this presumption. The ether-extracted, powdered muscle gave flourishing cultures which were maintained in subculture for three months and then discontinued. Doubtless muscle tissue from other fishes could have been used with similar results.

Experiments with some organic constituents of fish muscle

Whatever the nature of the constituents of fish muscle that are so important in the culture of *Volvox,* it is clear from the foregoing experiments that the proteins,

lipoids, and inorganic salts are unnecessary. This leaves carbohydrates (glycogen, etc.), nitrogenous extractives (creatine, urea, uric acid, etc.), non-nitrogenous extractives (lactic acid, inosite, etc.), pigments, enzymes, and growth-promoting factors, and perhaps other substances to be considered. It seemed most likely that one or more of the nitrogenous and non-nitrogenous extractives might support the continuous culture of *Volvox*. Therefore, experiments were undertaken in which lactic acid, urea, creatine, and uric acid were tested singly and in combination in various concentrations from 5 mg./l. to 100 mg./l. of local spring water, the usual amount of ferric chloride being added to all media. None of the cultures, however, was successful. In several instances adult colonies released daughter colonies, but these gradually lost their healthy green color and soon deteriorated. In a medium composed of urea, peptone, and lactic acid (5 mg./l. each) as many as three generations were produced before death occurred.

A duplicable culture medium for Volvox aureus

An analysis of Huckleberry spring water by D. M. Pace some fifteen years ago (personal communication) showed it to have the following inorganic composition:

Na_2SiO_3	12 mg./l.
NaCl	12 mg./l.
Na_2SO_4	6 mg./l.
$CaCl_2$	6.5 mg./l.
$MgCl_2$	3.5 mg./l.
$FeCl_3$	2–3 mg./l.

An artificial spring water was made up according to the above formula except that sufficient ferric chloride solution was added to give a concentration of this salt of 5 mg./l. Using ether-extracted, powdered sunfish muscle (200 mg./l.), culture medium was prepared according to the same method previously described for the fishmeal and fish muscle media. Cultures of *Volvox* have been maintained on this medium in a flourishing state for a period of four months, showing no evidence of decrease in vitality. These cultures were in every respect equal to cultures of the organism secured with commercial fishmeal extract.

Although fishmeals may vary somewhat in composition depending on their source and mode of manufacture, the powdered sunfish muscle prepared as herein described may be expected to have a constant composition wherever prepared. This method for the culture of *V. aureus*, therefore, is readily duplicable.

Discussion

Results of the foregoing experiments fail to confirm those of Uspenski and Uspenskaja (1925), Bold (1936), and Johansen (1940) who have cultured *Volvox* in inorganic salt solutions in the absence of organic matter. The findings, however, are in agreement with those of S. O. Mast (personal communication) and his students who were unable after many experiments to secure permanent cultures of *Volvox* in Uspenski's, Knop's, and other synthetic media. Pringsheim (1930) was evidently unable to maintain *V. aureus* and *V. globator* for any length of time in the Uspenski medium.

It is difficult to believe that failure to culture *Volvox* in the Uspenski medium was a result of impurities in the salts or the kind of glassware used in the present experiments. The salts were of the same degree of purity as those generally employed in the culture of protozoa and algae. The salts used in the preparation of artificial Huckleberry spring water apparently had no toxic effect.

Various investigators, notably Pringsheim (1930), have called attention to the deleterious effects produced on organisms by the kind of glass of which culture dishes are composed. He believed that unfavorable results were due to soluble constituents of the glass causing the medium to become more alkaline. Although ordinary finger bowls were used in the present experiments, no deleterious effect of the glass was observed. Successful cultures on fishmeal and fish muscle extract media were secured in the same glass finger bowls in which Uspenski's medium failed to support continuous growth and reproduction of *Volvox*.

Nor can failure to secure permanent cultures in the Uspenski medium be ascribed to an unfavorable pH. The initial pH was 7.6 (identical with that given by Uspenski and Uspenskaja) and remained at that value over a period of three weeks. That this is a favorable reaction is shown by the fact that the pH of freshly prepared fishmeal extract medium is 7.5–7.6, around which value it fluctuates from day to day gradually rising to a final pH of 8.0–8.2.

Mainx (1929) and Pringsheim (1930) secured rich cultures of *Volvox* in decoctions prepared from garden soil. Soils, however, vary widely in composition and reaction so that this method is not readily duplicable. Fishmeal medium, on the other hand, can be made up fairly accurately and is more readily reproducible. This is even more true of the fish muscle extract prepared with artificial Huckleberry spring water and the muscular tissue of the sunfish or butterfish.

The experiments of the present work show that the nutritional requirements of *Volvox aureus* may be more complex than is indicated by the work of Uspenski and Uspenskaja (1925) and that inorganic salts of spring water as well as organic substances of fish muscle are essential if this organism is to be maintained in culture for any length of time. What these salts and organic substances are, remains for further work to show; but it is quite possible that water-soluble growth promoting substances play an important rôle.

Summary

1. *Volvox aureus* Ehrenberg has been maintained in rich clone cultures for eighteen months without observable decrease in vitality on a medium prepared from spring water, commercial fishmeal, and ferric chloride.

2. The presence of iron in the medium was found to be essential to the continuous culture of *Volvox*. This was added as ferric chloride. Cultures lacking iron died out in about six weeks.

3. Either local spring water or Huckleberry spring water served satisfactorily in the preparation of fishmeal extract medium, but tap water and distilled water could not be substituted for these.

4. Spring water media containing peptone, beef extract, milk, or urine in various concentrations would not support continuous growth and reproduction of *Volvox*.

5. Uspenski's medium and modified Knop's solution were found to be of little value in the culture of *Volvox*.

6. Powdered muscle of the marine butterfish (*Poronotus triacanthus* Peck) or the fresh-water sunfish (*Lepomis gibbosus* Linnaeus), with lipoids extracted, could be substituted for commercial fishmeal, while skin and bone could not.

7. Spring water containing only the inorganic salts of fishmeal, or only the proteins, failed to support the culture of *V. aureus;* but spring water containing the cold water extractives and ferric chloride (one-half cc. of a one per cent solution of $FeCl_3 \cdot 6H_2O$/l.) gave excellent cultures which probably could have been carried indefinitely.

8. *V. aureus* could not be cultured in media containing lactic acid, urea, creatine, uric acid, or a combination of urea, peptone, and lactic acid in the several concentrations used.

9. A readily duplicable method for the culture of *V. aureus* is described.

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